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Poliovirus Infection Induces Apoptosis in CaCo-2 Cells

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The effects of poliovirus infection in CaCo-2 cells, a human enterocyte-like cell line are described. Infected cells were examined by a combination of techniques, including optical and electron microscopy, cytofluorimetric analysis of DNA content, and DNA agarose gel electrophoresis. Results obtained by the different experimental approaches demonstrate that poliovirus infection in enterocyte-like cells can result in an apoptotic process. ***J. Med. Virol. 59:122–129, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: apoptosis; poliovirus; in vitro

INTRODUCTION

Poliovirus are non-enveloped icosahedral single-stranded RNA viruses belonging to the *Picornaviridae* family. Viral infection in cultured cells is associated with a characteristic cytopathic effect: cells round up, shrink, display marked nuclear pycnosis, and become refractile [Melnick, 1996]. Poliovirus infection can result in cell death.

It has been generally accepted that there are two distinct, mutually exclusive pathways of cell death: necrosis and apoptosis [Darzynkiewicz et al., 1997]. Necrosis is accidental death [Majno and Joris, 1995] characterised by rupture of the plasma membrane and release of cytoplasmic constituents. A typical feature of necrosis is an inflammatory reaction in the tissue that often leads to scar formation [Wyllie, 1992; Majno and Joris, 1995]. Programmed cell death (PCD), or apoptosis, is defined as a physiological cell suicide process alternative to necrosis and is characterised morphologically, in vivo, by DNA aggregation and by the formation of membrane-enclosed apoptotic bodies and blebs containing well preserved organelles [Wyllie, 1981; Kerr et al., 1984; Wyllie et al., 1984; Malorni and Donelli, 1992; Schwartzman and Cidlowski, 1993]. It has been hypothesised that PCD may play a central role in embryonic development, as well as in human malignancies [Smith et al., 1989; Evans, 1993], and in the pathogenesis of several infectious diseases [Zychlinsky, 1993]. In addition, more recently, studies investigating virus-associated lytic processes have been carried out and an important role for the apoptotic cell death pathway in numerous viral infections has been found [Desmet, 1988; Ameisen and Capron, 1991; Cohen, 1991; Groux et al., 1991; Hugin et al., 1991; Morey et al., 1993; Takizawa et al., 1993; Hinshaw et al., 1994; Vasconcelos and Lam, 1994; Yamada et al., 1994; Esolen et al., 1995; Tropea et al., 1995; Bagetta et al., 1996; Superti et al., 1996; Mastino et al., 1997]. In particular, different viruses (belonging to various families) have been demonstrated to induce, or to prevent, apoptosis [Jeurissen et al., 1992; McCabe and Orrenius, 1992; Meyaard et al., 1992; Rojko et al., 1992; Thompson, 1995]. However, general rules for viral infection-related cell death have still to be established.

The present study focuses on the possible relationship between poliovirus infection and apoptosis, with the aim of gaining further insights into the cellular changes occurring in poliovirus-infected cells. As intestinal cells represent an initial site of viral replication, we exploited the in vitro model of CaCo-2 cells. This enterocyte-like cell line forms relatively well-differentiated epithelial monolayers spontaneously in vitro, exhibiting several characteristics of human intestinal epithelial cells [Fogh et al., 1977; Rousset, 1986] and, moreover, is highly permissive to poliovirus infection [Reigel, 1985; Tucker et al., 1993].

Results are described showing that viral infection causes nuclear fragmentation due to chromatin condensation and also induces fragmentation of chromosomal DNA into oligonucleosomes, thus indicating that poliovirus infection triggers the development of apoptosis in intestinal cells.

MATERIALS AND METHODS

Cells and Virus

The human colon adenocarcinoma cell line CaCo-2 was obtained from American Type Culture Collection (ATCC; Rockville, MD). Poliovirus type 1 (Mahoney strain), grown in Vero cells, was kindly provided by Dr. Maurizio Divizia (University "Tor Vergata", Rome).

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Ultraviolet (UV) Irradiation of Virus

Virus was inactivated by UV irradiation according to Marchetti et al. [1992]. The UV-treated virus preparation was tested in CaCo-2 cell cultures and no cytopathic effect was obtained after a 48-hr incubation.

Subcellular Analysis of Infected Cells

CaCo-2 cells, grown for 48 hr at 37°C, were infected with poliovirus (multiplicity of infection (m.o.i.) of 1 and 10 plaque-forming units [p.f.u.]/cell). After 1 hr adsorption at 37°C, the cells were washed with modified Eagle's medium (MEM) to remove the inoculum and incubated at 37°C for different times (8, 16, and 24 hr). DNA fragmentation, cell necrosis, and cell viability were determined in virus-infected and "mock-infected" cells. "Mock-infected" cells were control cells incubated with supernatants from lysates of uninfected Vero cells (prepared like the virus preparation) for 1 hr at 37°C, washed, and then incubated at 37°C for the same intervals of time as described for viral infection.

Cells, stained with acridine orange (100 μg/ml)-ethidium bromide (100 μg/ml) solution, were examined under UV illumination (Nikon). In all experiments, each sample was analysed in triplicate by counting at least 200 or more cells for replicate. The characteristics of the cells were recorded according to the colour and the structure of the chromatin [Duke and Cohen, 1992).

Detection of Apoptosis by Propidium Iodide-Staining and Flow Cytometry

After 8, 16, and 24 hr of infection with 1 and 10 p.f.u./cell, mock-infected and virus-infected cells, harvested and pooled with the supernatants, were stained with propidium iodide (PI) and analysed by flow cytometry as described previously [Superti et al., 1996]. About 20,000 events/sample were accumulated and the list mode data analysed by Lysis II-C32 Becton Dickinson software.

Transmission Electron Microscopy (TEM)

CaCo-2 cells infected with poliovirus (50 p.f.u./cell) and control cells were fixed at different times postinfection (p.i.) (4, 8, and 16 hr) and processed for TEM as described previously [Superti et al., 1996].

DNA Gel Electrophoresis

To separate high molecular weight chromatin from the nucleosomal DNA fragments, virus-infected (1 and 10 p.f.u./cell) and mock-infected cells were processed according to Malorni et al. [1993]. DNA was subjected to electrophoresis on 1.5% agarose gel. An *Eco*147I and *Pvu*I digest of pMLX DNA provided molecular weight standards. After electrophoresis, the gel was stained with ethidium bromide and photographed.

Indirect Immunofluorescence

Viral antigen synthesis was evaluated by indirect immunofluorescence. The cells were treated with hyperimmune human anti-poliovirus serum, kindly provided by Dr. Maurizio Divizia (University "Tor Vergata", Rome), and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-human gammaglobulin antibodies (Sigma Chemical Co.). Cells were viewed through a Leitz Dialux fluorescence microscope. Six hundred cells were examined for each sample and the results were expressed as the percentage of infected cells.

Statistical Analysis

Statistical analysis was performed using the Student's *t*-test for unpaired data. Data were expressed as the mean ± SD and $P < .05$ were considered significant.

RESULTS

Cytological Analyses

When observed by means of a bright field light microscopy, CaCo-2-infected cells underwent a number of morphological changes with respect to control cells. In particular, uninfected cells appeared to grow as cell clusters and in patches, whereas the progression of infection-induced cells to round up, shrink, detach from the substrate, and, finally to float in the medium (data not shown).

The percentage of apoptotic, necrotic, and viable cells at the different times of infection was evaluated on the entire cell population (adherent and detached cells). Results obtained are shown in Figure 1. Typical apoptotic cells increased as the time of infection progressed. At 8, 16, and 24 hr p.i. with an m.o.i of 1, the percentage of apoptotic cells was 8%, 25%, and 60%, respectively (Fig. 1a). Apoptotic indexes correlated well with the percentage of infected cells that, at the same intervals of time, were 40%, 70%, and 100%, respectively (data not shown). The highest levels of apoptosis were observed with an m.o.i. of 10, which, in the same time intervals, induced apoptotic death in 10%, 42.5%, and 65% of the cells, respectively. In these experimental conditions, 70%, 90%, and 100% of fluorescent cells were observed (data not shown). The percentage of apoptotic cells in negative controls at 8, 16, and 24 hr did not exceed 3%, 5%, and 7.5%, respectively. Hence, a relationship between apoptosis and virus concentration seemed to occur so that at 16 and 24 hr p.i., cells infected with an m.o.i of either 1 or 10, displayed statistically significant differences in the apoptotic indices as compared with mock-infected cells (P values < .0001). In some experiments, 16 and 24 hr after infection, the assay was undertaken separately on attached and detached cells. The chromatin alterations appeared to be more frequent in floating cells than in substrate-adhering cells (data not shown). The incidence of necrosis induction did not increase as much as apoptosis (Fig. 1b), reaching the percentage of 27.5% after 24 hr of infection with 10 p.f.u./cell. Results obtained on cell viability are shown in Figure 1c. Poliovirus infection affects cell viability with a dose- and time-dependent relationship. After 24 hr, the percentage of viable mock-infected cells was 85%, whereas the percentage of viability in infected cells was 65% and 40%

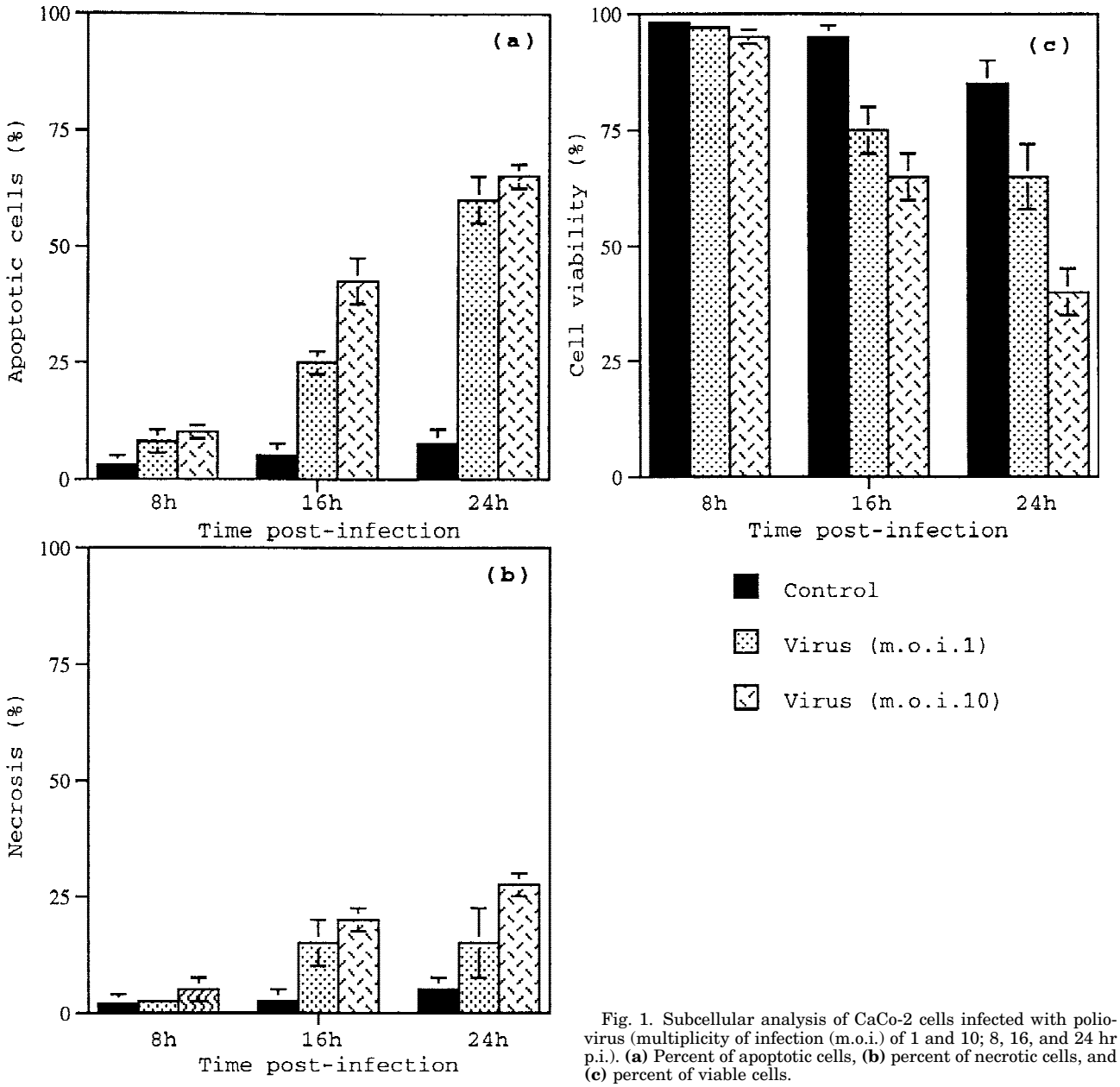

Fig. 1. Subcellular analysis of CaCo-2 cells infected with poliovirus (multiplicity of infection (m.o.i.) of 1 and 10; 8, 16, and 24 hr p.i.). **(a)** Percent of apoptotic cells, **(b)** percent of necrotic cells, and **(c)** percent of viable cells.

with the m.o.i. 1 and the m.o.i. 10, respectively. In these experiments, additional controls were performed by utilizing UV-inactivated virus. This virus was added to the cells in the same experimental conditions used for viral infection. The apoptotic rates induced by UV-treated virus were similar to those observed in uninfected cells as, at 24 hr p.i. with an m.o.i. of 10, apoptosis was observed in fewer than 10% of cells (data not shown).

Time Course Ultrastructural Features

Mock-infected and virus-infected CaCo-2 cells were analysed in their ultrastructural features. Poliovirus-infected cells (4, 8, and 16 hr p.i.) are shown in Figure 2. At 4 hr p.i., CaCo-2-infected cells did not display detectable morphological changes (Fig. 2a). However, features typical of programmed cell death processes were found at 8 and 16 hr p.i. After 8 hr of infection (Fig. 2b), changes in the density and distribution of chromatin, which condensed peripherally into a crescent-shaped mass, and dilatation of nuclear cisternae were observed. At this time, morphological signs of cell injury, such as intracytoplasmic vacuolation, were also detected. Finally, 16 hr after infection, cells displayed the typical markers of apoptotic cell death such as nuclear segmentation and cytoplasmic derangement (Fig. 2c). Moreover, numerous viral particles were found in the cytoplasm (Fig. 2d).

Detection of Apoptosis by PI Staining and Flow Cytometry

Results obtained by PI staining were similar to those obtained with other intercalating DNA dyes (such as

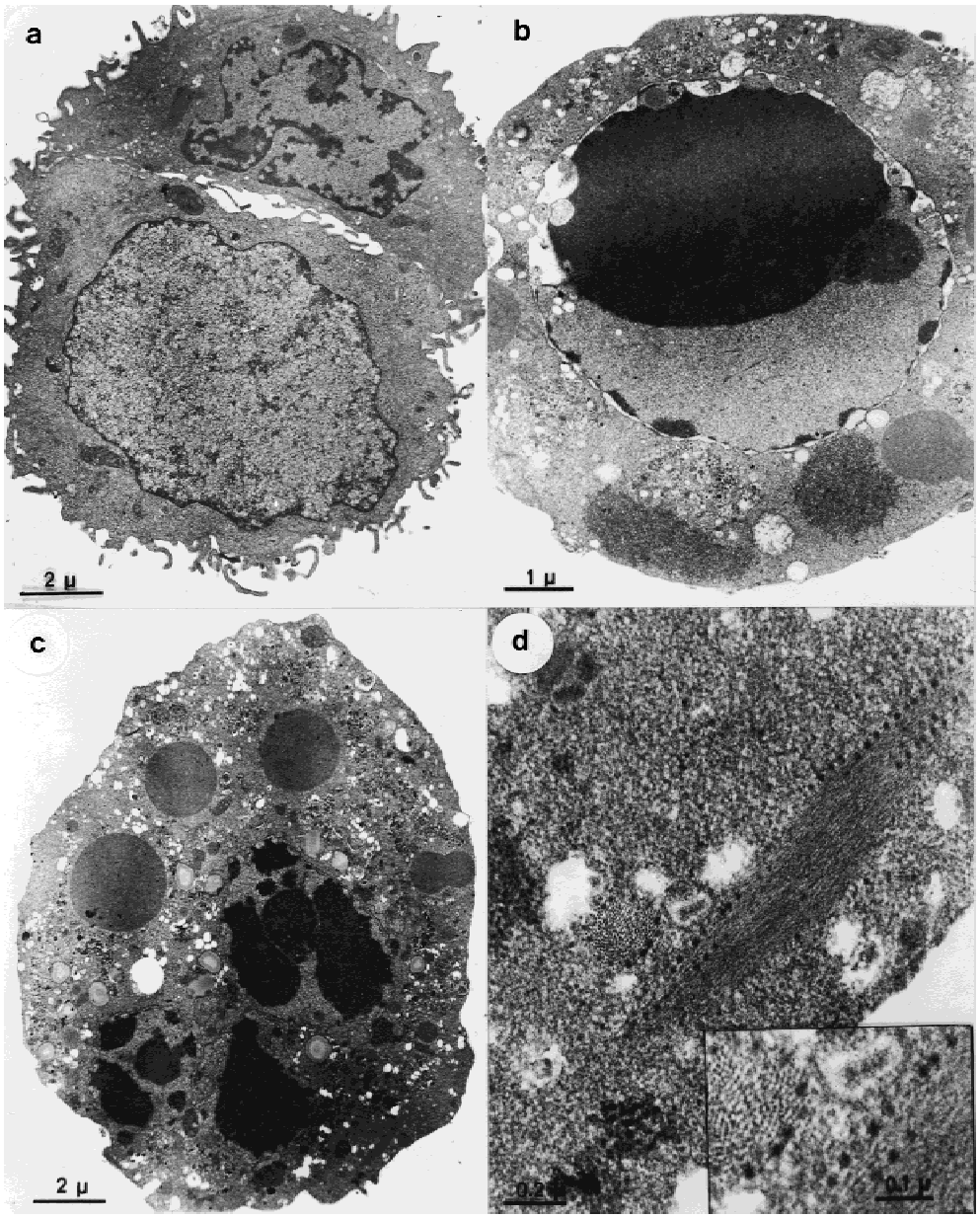

Fig. 2. Transmission electron micrographs of poliovirus-infected cells. **(a)** At an early phase of infection (4 hr postinfection [p.i.]), neither morphological changes or viral particles are detectable in infected cells. **(b)** At 8 hr of infection, cells exhibit chromatin condensation, dilation of perinuclear cisternae and intracytoplasmic vacuolation, and cytoplasmic electron-dense inclusions corresponding to replication complexes are also observed. **(c)** At 16 hr p.i., cells display typical markers of apoptosis such as nuclear segmentation and cytoplasmic derangement. **(d)** A higher magnification of a cytoplasmic portion of Figure 3c revealing numerous viral particles closely associated with tangentially and longitudinally cut intermediate filament network (see also inset).

acridine orange and ethidium bromide). As shown in Figure 3, the induction of apoptosis in Caco-2 cells was a time- and virus concentration-dependent event. In fact, 16 hr and 24 hr after infection with 1 p.f.u./cell, apoptosis was detected in 30% and 44% of cells, respectively, and the infection of CaCo-2 cells with an m.o.i. of 10 for 24 hr induced the apoptotic process in a significant proportion of the cell population (57%). The effect of zinc, which is known to inhibit DNA fragmentation occurring in apoptosis [Cohen and Duke, 1984] was examined. Cells were infected with an m.o.i. of 1 and 10 and $ZnSO_4$, at a concentration of 0.1 mM, was added to the medium just after infection. After 24 hr incubation at 37°C, mock-infected and virus-infected cells were analysed by flow cytometry. As shown in Figure 4, $ZnSO_4$ blocked the nuclear fragmentation induced by viral infection as the percentage of apoptotic cells in zinc-treated poliovirus-infected cells was similar to that observed in mock-infected cultures (5%, 7.5%, and 3%, respectively).

DNA Fragmentation

The analysis of DNA from mock-infected and virus-infected cells by agarose gel electrophoresis is shown in

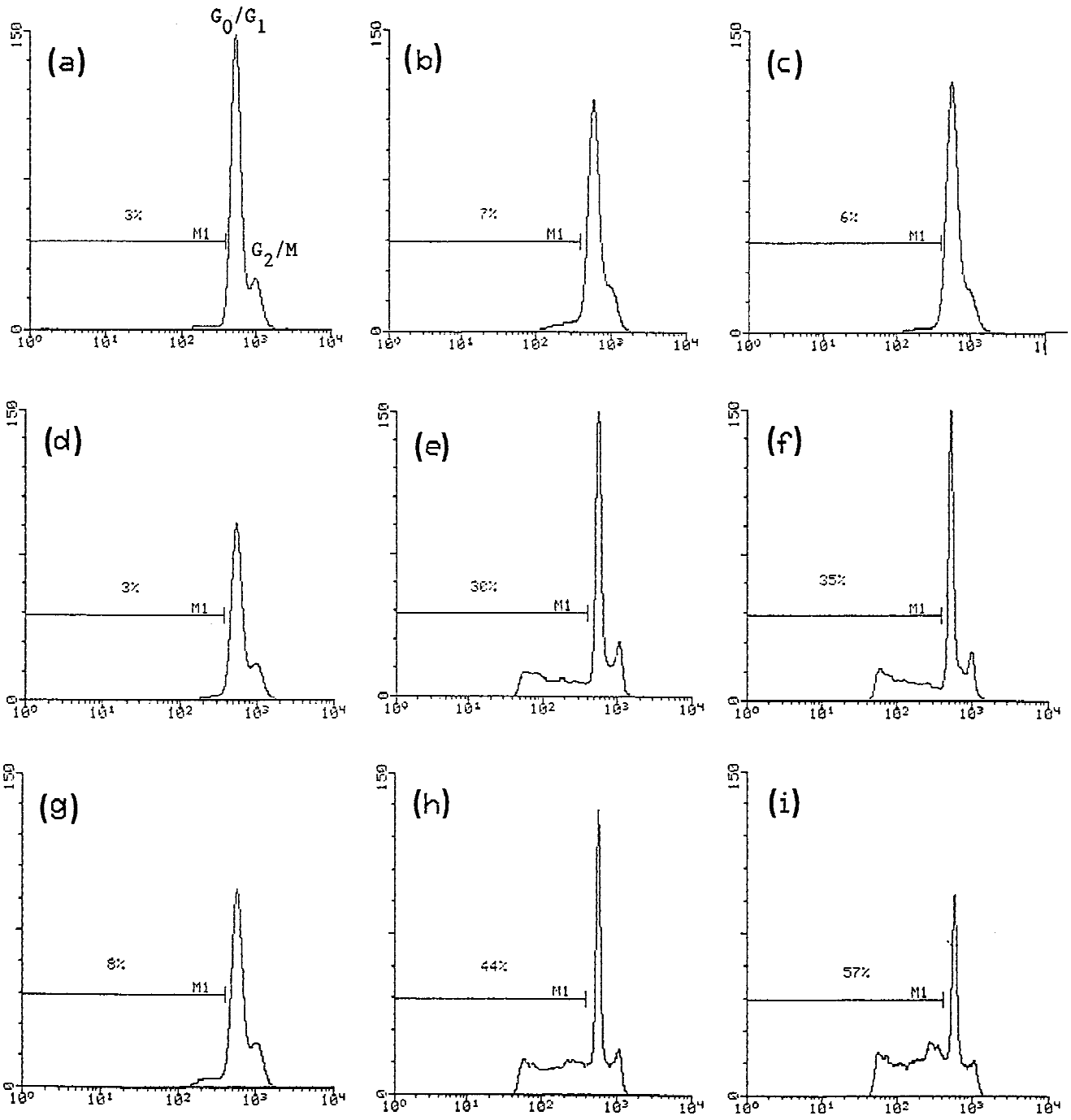

Fig. 3. Flow cytometric analysis of DNA content of propidium iodide (PI)-stained CaCo-2 cells. The percentage of apoptotic cells was obtained by calculating the percentage of the cell population showing a DNA content lower than G0/G1 cells. DNA histograms: **(a, d, g)** mock-infected cells, **(b, e, h)** virus-infected cells, m.o.i. 1, and **(c, f, i)** virus-infected cells, m.o.i. 10. Analyses were performed at different times post-infection: (a, d, g) 8 hr, (b, e, h) 16 hr, and (c, f, i) 24 hr.

Figure 5. DNA fragmentation at the internucleosomal level resulting in a characteristic ladder was evident in poliovirus-infected cells. The amount of DNA fragmentation exhibits a dose-dependent relationship, being more evident in cells infected with 10 p.f.u./cell. As expected, the ladder was undetectable in uninfected control cells. The effect of zinc on poliovirus-mediated apoptosis was also studied. As shown in the figure, $ZnSO_4$ treatment was able to prevent DNA fragmentation induced by viral infection.

DISCUSSION

The effect of poliovirus replication in CaCo-2 cells was studied and the results obtained demonstrate that viral infection induces PCD in intestinal cells. Acridine

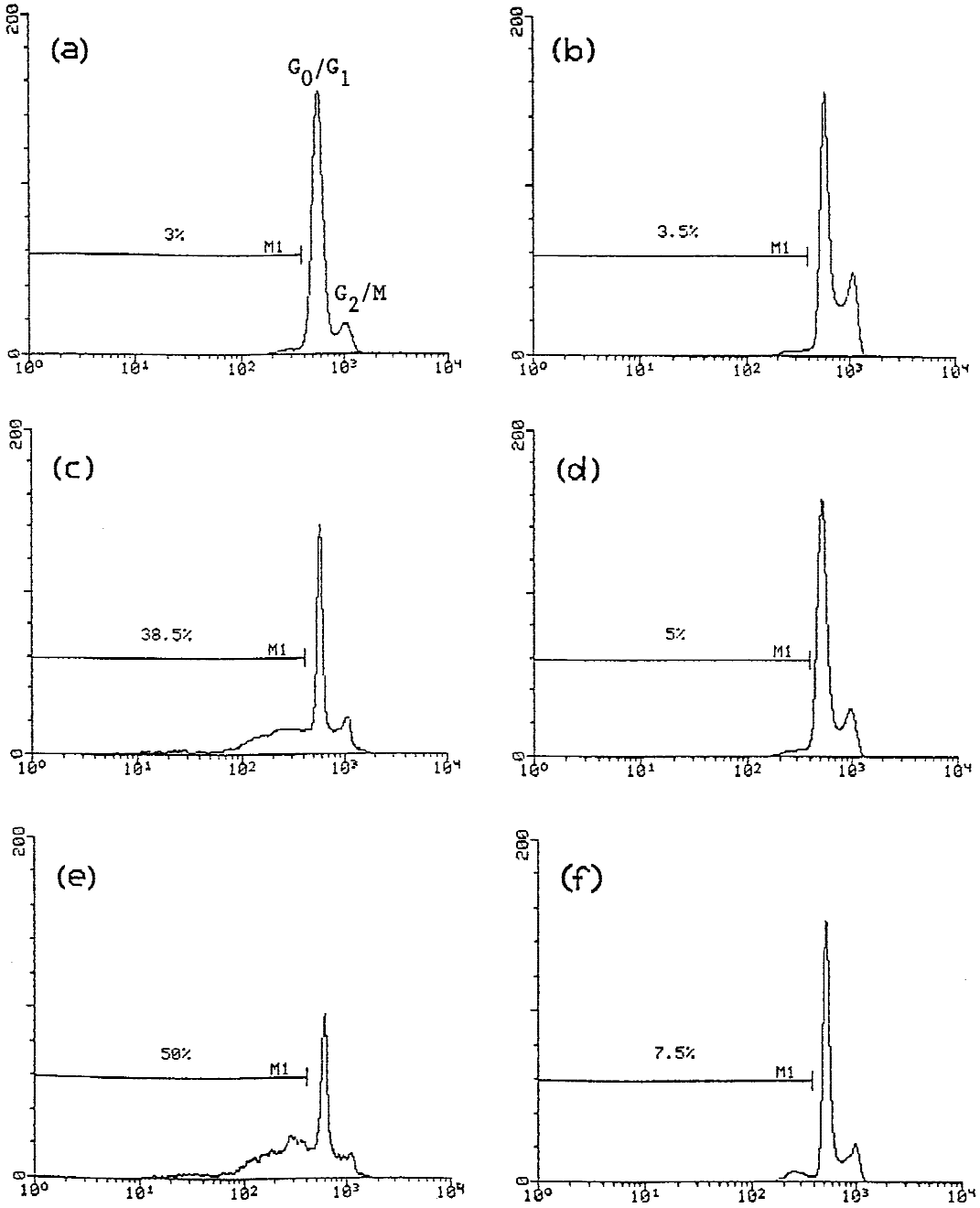

Fig. 4. Flow cytometric analysis of DNA content of propidium iodide (PI)-stained CaCo-2 cells: effect of $ZnSO_4$ on nuclear fragmentation in poliovirus-infected cells. $ZnSO_4$ (0.1 mM) was added to the cells just after infection. The percentage of apoptotic cells was obtained by calculating the percentage of the cell population showing a DNA content lower than G0/G1 cells. DNA histograms: **(a, b)** mock-infected cells, **(c, d)** virus-infected cells, m.o.i. 1, and **(e, f)** virus-infected cells, m.o.i. 10. (b, d, f) $ZnSO_4$-treated cells. Analyses were performed 24 hr postinfection.

orange-ethidium bromide staining showed a close relationship between apoptosis and viral replication and also indicated PCD as a virus concentration-dependent process. Also, with the progression of infection, the apoptotic indices became increasingly higher than the necrotic indices.

Ultrastructural analysis by TEM confirmed the results obtained by morphological assays with fluorescent dyes and demonstrated that peculiar features could be found in CaCo-2 cells undergoing apoptosis. Typical signs of the apoptotic process began to appear at 8 hr p.i. when electron-dense structures, corresponding to poliovirus replication complexes, were also observed. As cell death progressed, complete viral particles were seen in the cytoplasm (16 hr p.i.) of infected cells. In the latest phases of PCD (24 hr p.i.), virions were not detectable in apoptotic infected cells (data not shown). Poliovirus-infected CaCo-2 cells showed cyto-

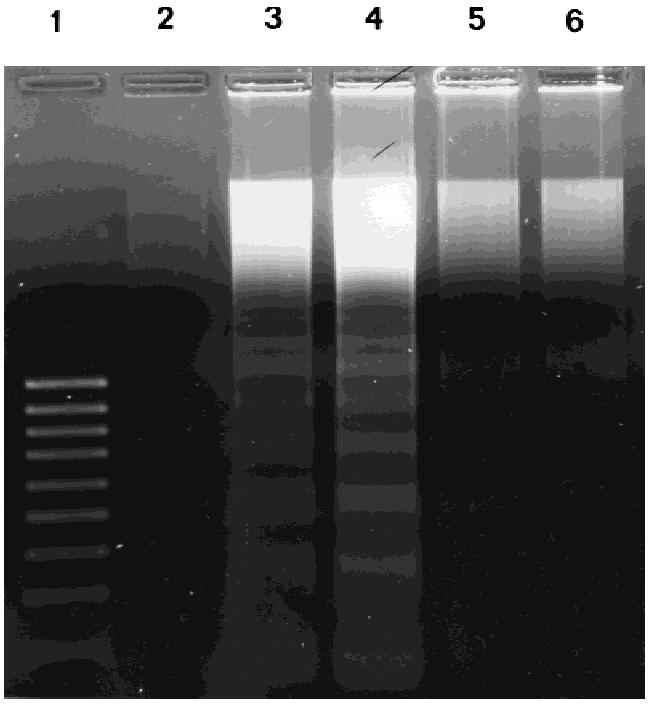

Fig. 5. Agarose gel electrophoresis showing DNA degradation (ladder pattern) of poliovirus-infected cells. Lane 1, M.W. marker of *Eco*147I and *Pvu*I digest of pMLX DNA; lane 2, uninfected cells; lane 3, poliovirus infected cells (m.o.i. 1); lane 4, poliovirus infected cells (m.o.i. 10); lane 5, poliovirus infected cells (m.o.i. 1) treated with $ZnSO_4$ (0.1 mM) just after infection; lane 6, poliovirus infected cells (m.o.i. 10) treated with $ZnSO_4$ (0.1 mM) just after infection. Results are representative of three independent experiments.

plasmic changes, such as intracytoplasmic vacuolation, typical of productive infection. In fact, previous ultrastructural studies carried out on different productively infected cell systems have described several cytoplasmic alterations [Dales et al., 1965; Bienz et al., 1987; Tolskaya et al., 1995; Schlegel et al., 1996]. Contrary to the observations of Tolskaya et al. [1995] in HeLa cells, in our cell system productive infection was characterised by both cytoplasmic and nuclear alterations.

The results from FACScan analysis by PI staining of infected cells indicated further that poliovirus infection induced chromosomal margination and separation. In fact, in the late stages of infection, about 60% of infected cells displayed DNA degradation.

These data were also confirmed by internucleosomal degradation (laddering) of chromosomal DNA obtained by gel electrophoresis assay. Infected cells treated with 0.1 mM $ZnSO_4$, which is known to inhibit the internucleosomal DNA fragmentation occurring in apoptosis, showed no signs of DNA degradation both in FACScan or in gel electrophoresis analyses.

Concerning the *Picornaviridae* family, apoptosis has been demonstrated with Theiler's murine encephalomyelitis virus in in vivo infection [Tsunoda et al., 1997] and for poliovirus in nonpermissive in vitro infection [Tolskaya et al., 1995, 1996]. Tolskaya et al. [1995] reported that poliovirus infection in a subline of HeLa-S3 cells (HeLA-B cells) turned on two oppositely directed sets of reactions: an apoptotic reaction, developing under nonpermissive conditions, and an antiapoptotic activity, occurring upon permissive infection. The current data were partially in disagreement with these observations as it must be considered, that in different cell systems, the interplay of cellular apoptosis-promoting and apoptosis-suppressing functions is controlled physiologically. In CaCo-2 cells, highly permissive to either viral replication or viral isolation from clinical material [Reigel, 1985; Tucker et al., 1993], poliovirus infection interferes with this cellular control by turning the balance toward cell death by apoptosis. It can be hypothesised that, during in vivo infection, poliovirus induces the apoptotic process to favour viral spread from intestinal cells in the absence of inflammatory reaction.

Poliovirus-induced apoptosis in CaCo-2 cells could be linked to the activation of several mechanisms such as the modulation of intracellular calcium concentration. It has been reported previously that intracellular calcium concentration greatly increases during the course of poliovirus infection [Irurzun et al., 1995]. Thus, virus cytopathic effect could be mediated by an increase in intracellular Ca^{++} induced by the synthesis of a viral product, the 2BC protein. This protein is responsible for cell permeabilization to calcium, even though it is still not clear whether this permeabilization is achieved by a direct effect of 2BC on the plasma membrane or by an indirect effect on a cellular protein, such as a calcium channel [Aldabe et al., 1997]. As disturbances in intracellular Ca^{++} homeostasis are linked to the onset of PCD in several cell systems [Nicotera et al., 1992], it is likely that apoptosis induced by poliovirus could be a Ca^{++}-mediated process. Moreover, the prevention of apoptosis by zinc treatment of infected cells suggests the involvement of calcium in poliovirus-mediated apoptosis of CaCo-2 cells.

Further studies on the role of calcium in poliovirus infection are needed because the data suggest that apoptosis should be considered as part of the cell lytic process induced by poliovirus.

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